



Published in final edited form as:

Arch Toxicol. 2016 March ; 90(3): 551–557. doi:10.1007/s00204-015-1489-6.

Quantification of tetrabromo benzoic acid and tetrabromo phthalic acid in rats exposed to the flame retardant Uniplex FPR-45

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Abstract

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Disclaimer: The animal research described in this article has been reviewed by the National Health Environmental Effects Research Laboratory, U.S. Environmental Protection Agency, and approved for publication. Approval does not signify that the contents necessarily reflect the views and policies of the Agency nor does mention of trade names or commercial products constitute endorsement or recommendation for use. The findings and conclusions in this report are those of the authors and do not necessarily represent the views of the Centers for Disease Control and Prevention.

Electronic supplementary material The online version of this article (doi:10.1007/s00204-015-1489-6) contains supplementary material, which is available to authorized users.

Conflict of interest The authors declare they have no competing financial or other conflicts of interests.

The first withdrawal of certain polybrominated diphenyl ethers flame retardants from the US market occurred in 2004. Since then, use of brominated non-PBDE compounds such as bis(2-ethylhexyl)-2,3,4,5-tetrabromophthalate (BEH-TEBP) and 2-ethylhexyl-2,3,4,5-tetrabromobenzoate (EH-TBB) in commercial formulations has increased. Assessing human exposure to these chemicals requires identifying metabolites that can potentially serve as their biomarkers of exposure. We administered by gavage a dose of 500 mg/Kg bw of Uniplex FRP-45 (>95 % BEH-TEBP) to nine adult female Sprague–Dawley rats. Using authentic standards and mass spectrometry, we positively identified and quantified 2,3,4,5-tetrabromo benzoic acid (TBBA) and 2,3,4,5-tetrabromo phthalic acid (TBPA) in 24-h urine samples collected 1 day after dosing the rats and in serum at necropsy, 2 days post-exposure. Interestingly, TBBA and TBPA concentrations correlated well ($R^2 = 0.92$). The levels of TBBA, a known metabolite of EH-TBB, were much higher than the levels of TBPA both in urine and serum. Because Uniplex FRP-45 was technical grade and EH-TBB was present in the formulation, TBBA likely resulted from the metabolism of EH-TBB. Taken together, our data suggest that TBBA and TBPA may serve as biomarkers of exposure to non-PBDE brominated flame retardant mixtures. Additional research can provide useful information to better understand the composition and in vivo toxicokinetics of these commercial mixtures.

Keywords

Flame retardant; EH-TBB; BEH-TEBP; Bis-(2-ethylhexyl)-2,3,4,5-tetrabromophthalate; 2-Ethylhexyl-2,3,4,5-tetrabromobenzoate; Tetrabromo benzoic acid; Tetrabromo phthalic acid

Introduction

Polybrominated diphenyl ethers (PBDEs) found in Penta-BDE and Octa-BDE technical mixtures were produced as flame retardants in the US market until their phase-out in 2004 (US EPA 2013). Since then, bis(2-ethylhexyl)-2,3,4,5-tetrabromophthalate (BEH-TEBP) and 2-ethylhexyl-2,3,4,5-tetrabromobenzoate (EH-TBB) have been introduced as replacements for Penta-BDE used as an additive in furniture foam and other products (US EPA 2014; Bergman et al. 2012). BEH-TEBP and EH-TBB are used in combination with other compounds in non-PBDE flame retardants such as Firemaster 550, Firemaster BZ-54, and CN-2065 (US EPA 2014; Carignan et al. 2013; Bearn et al. 2010). Because of the increased use of non-PBDE flame retardants in consumer products, BEH-TEBP and EH-TBB have been detected in household dust (Ali et al. 2011, 2012; Stapleton et al. 2008), and exposure to these compounds in the general population is likely to occur.

Assessing human exposure to BEH-TEBP and EH-TBB requires identifying metabolites that can potentially serve as biomarkers of exposure. In previous studies using human and rat tissues, 2,3,4,5-tetrabromo benzoic acid (TBBA) was identified as an in vitro metabolite of EH-TBB (Roberts et al. 2012). Furthermore, TBBA was detected in 72.4 % of urine samples from 64 North Carolina adults (Hoffman et al. 2014) and in 27 % of adults and 70 % of children among 21 US mother–toddler pairs (Butt et al. 2014). However, metabolites of BEH-TEBP have not been identified yet. To fill in this gap, we performed a proof-of-

concept study using Sprague–Dawley rats to identify and quantify urinary and serum metabolites of a flame retardant mixture whose main component (>95 %) is BEH-TEBP.

Materials and methods

Chemicals

Technical grade Uniplex FRP-45 (>95 % BEH-TEBP) was obtained from Unitex Chemical Corporation (Greensboro, NC). 2,3,4,5-Tetrabromo phthalic acid (TBPA) was purchased from TCI (Tokyo, Japan). TBBA was purchased from Small Molecule Synthesis Facility (Duke University, Durham, NC). Ammonium acetate and di-2-ethylhexyl phthalate (DEHP) were purchased from Sigma-Aldrich Laboratories, Inc. (St. Louis, MO). Acetonitrile (HPLC grade) was purchased from Burdick and Jackson (VWR, Radnor, PA). β -Glucuronidase (*Escherichia coli-K12*) was purchased from Roche Biomedical (Mannheim, Germany). Mono-2-ethyl-5-carboxypentyl phthalate (MECPP) and $^{13}\text{C}_6$ -MECPP were purchased from CanSyn (Ontario, Canada). Mono-2-ethyl-5-hydroxyhexyl phthalate (MEHHP) and D_4 -MEHHP were purchased from ADM (Teltow, Germany). EH-TBB (50 $\mu\text{g/mL}$ in toluene) and BEH-TEBP (50 $\mu\text{g/mL}$ in toluene) were purchased from Wellington Laboratories (Ontario, Canada). All chemicals and standard materials were used without further purification.

Animal experiments

The animal experiments were conducted at the Reproductive Toxicology Animal Facility of the U.S. Environmental Protection Agency (EPA) in Research Triangle Park, NC. Twelve female Sprague–Dawley rats (Harlan Laboratories, Indianapolis, IN) aged ~60 days, weighing ~250 g, were housed individually in clear polycarbonate cages ($20 \times 25 \times 47 \text{ cm}^3$) with laboratory-grade pine shavings as bedding, with a 12:12 light/dark cycle (lights off at 7:00 p.m.), temperature of 20–22 °C, and 45–55 % relative humidity. The rats were provided with filtered (5 μm) water and NTP 2000 rat chow ad libitum. After 1 day of acclimation, the rats were weighed, and one dose of 500 mg Uniplex FRP-45 per kilogram of body weight (bw) in corn oil was administered orally to nine rats by gavage. Three other rats used as controls received only corn oil.

For each of the nine rats, 24-h urine samples were collected 1 day before and 24 h after administration of corn oil with or without Uniplex FRP-45. Approximately 48 h after administration, the 12 (including the three “controls”) rats were euthanized and serum was collected. The urine and serum samples were placed in polypropylene vials and frozen at –70 °C until shipped on dry ice to the National Center for Environmental Health laboratory at the Centers for Disease Control and Prevention (CDC). At CDC, the urine and serum samples were stored at –70 °C until analyzed. Specific gravity of the urine was measured at CDC using a hand-held refractometer (ATAGO, Bellevue, WA). The animal treatment protocol was reviewed and approved by the EPA Internal Animal Care and Use Committee.

Standards preparation

Stock standard solutions of MECPP, MEHHP, D_4 -MEHHP and $^{13}\text{C}_6$ -MECPP, TBBA, and TBPA were prepared in acetonitrile and stored at –70 °C in Teflon-capped amber glass

bottles until use. The working standards were prepared in 1:9 acetonitrile: water from dilutions of the stock solutions to create ten unique working standard solutions (Silva et al. 2007). The working standards were stored at -70°C in 5 mL cryovials until use.

Screening of Uniplex FRP-45

Solutions of EH-TBB (50 $\mu\text{g/mL}$ in toluene), BEH-TEBP (50 $\mu\text{g/mL}$ in toluene), DEHP (50 $\mu\text{g/mL}$ in toluene), and Uniplex FRP-45 (50 $\mu\text{g/mL}$ in toluene) were separately analyzed using a LECO Pegasus[®] HT TOF-MS detector coupled with an Agilent 6890 GC (LECO Corp., St. Joseph, MI, USA) configured with a 20 m \times 0.18 mm, 0.18 μ Rxi-5Sil MS column (Restek, Bellefonte, PA, USA). The carrier gas was helium at a constant flow of 0.6 mL/min. One microliter of sample was injected in splitless mode with the inlet at 280 $^{\circ}\text{C}$. The GC oven program was as follows: started at 90 $^{\circ}\text{C}$, held for 45 s, ramped at 50 $^{\circ}\text{C/min}$ to 135 $^{\circ}\text{C}$, then ramped at 25 $^{\circ}\text{C/min}$ to 350 $^{\circ}\text{C}$, and held for 5 min. The transfer line was maintained at 350 $^{\circ}\text{C}$. The ion source was maintained at 240 $^{\circ}\text{C}$, and data were acquired at ten spectra per second in positive ion mode. Data were processed with the instrument software ChromaTOF[®]. Retention times for DEHP, EH-TBB and BEH-TEBP were confirmed by injecting standard solutions. Mass transitions of $m/z = 149$, $m/z = 421$, and $m/z = 465$ were used for DEHP, EH-TBB, and BEH-TEBP, respectively (Supplementary data, Figure S1).

Standard solutions of EH-TBB (50 $\mu\text{g/mL}$ in toluene), BEH-TEBP (50 $\mu\text{g/mL}$ in toluene), DEHP (50 $\mu\text{g/mL}$ in acetonitrile), and Uniplex FRP-45 in acetonitrile were separately infused (5 $\mu\text{L/min}$) into a Q Exactive high-resolution mass spectrometer (ThermoFinnigan, San Jose, CA) and scanned for $[\text{TBB}]^{+}$ and $[\text{DEHP}]^{+}$ transitions in positive ion mode (Supplementary data, Figures S2 and S3).

Identification of Uniplex FRP-45 metabolites

The 24-h urine collected from the rats after dosing was diluted (1:10) with water and directly infused (5 $\mu\text{L/min}$) into a TSQ Vantage AM triple quadrupole mass spectrometer (ThermoFinnigan, San Jose, CA). All ions on Q1 from $m/z = 300$ to $m/z = 800$ in negative electrospray ionization (ESI) mode were scanned to identify brominated metabolites, including those resulting from the oxidation of the 2-ethylhexyl alkyl chain moiety as well as tri-, di-, or mono-brominated compounds. We did not identify brominated oxidative products or debrominated compounds. The fragmentation patterns of ESI Q1 full scan negative ion mass spectra of the major peaks with bromine mass spectral isotopic pattern were analyzed after HPLC separation using 0.1 M ammonium acetate:acetonitrile solvent gradient (Supplementary data Table S1). Q1 scans and mass spectral fragmentation analysis after HPLC separation were also performed for TBBA and TBPA standard solutions (1 mg/L, Fig. 1). Urinary and serum TBBA and TBPA were positively identified in dosed rats using authentic standards. Urine from the rats before dosing was used as control.

Quantification of TBPA, TBBA, MEHHP, and MECPP

Urine or serum from dosed rats (20 μL) and controls (100 μL) was processed by adding the labeled internal standard solution (100 μL) containing 4-methylumbelliferyl glucuronide, D₄-MEHHP and ¹³C₆-MECPP. The glucuronide conjugates were hydrolyzed by incubating

at 37 °C for 2 h with β -glucuronidase (Roche Biomedical, Mannheim, Germany). The level of 4-methylumbelliferone was evaluated to monitor the completion of the deconjugation (Silva et al. 2007). The spiked serum or urine was pretreated using a previously published method (Silva et al. 2007). Each processed sample was loaded onto a RP-18e chromolith pre-column (4.6 mm \times 5 mm, Merck KGaA, Germany), which served as a solid-phase extraction column, using 10 mM ammonium acetate. The column was rinsed with 10 mM ammonium acetate:acetonitrile (80:20) at a flow rate of 1 mL/min (Supplementary data, Table S1). The flow was reversed after 1 min, and the target analytes were back-eluted onto a Betasil Phenyl analytical column (3 μ m, 150 mm \times 2.1 mm, ThermoHypersil-Keystone, Bellefonte, PA), preceded by inline filters (2 μ m and 0.5 μ m, Upchurch Scientific, Oak Harbor, WA). The analytes were chromatographically resolved using a nonlinear 10 mM ammonium acetate (pH 6.7): acetonitrile solvent gradient (Supplementary data, Table = S1) and detected using tandem mass spectrometry in negative ion mode using specific precursor and product ions (Table 1). All analytes eluted as sharp peaks within 12 min. The method limits of detection (LOD), defined as $3S_0$, where S_0 is the standard deviation as the concentration approaches zero (Taylor 1987), were 0.4 ng/mL (TBPA, TBBA), and 0.2 ng/mL (MEHHP, MECPP). D₄-MEHHP was used as the internal standard for TBPA and TBBA (Table 2). For quantification of TBPA and TBBA, a 10-standard calibration curve of the peak area ratio of the analyte to D₄-MEHHP versus standard concentration was used. Calibration curves of TBBA and TBPA were linear with correlation coefficients >0.98 .

Results and discussion

We used Uniplex FRP-45, a commercial flame retardant technical mixture containing mainly ($>95\%$) BEH-TEBP. Because information on the composition of the remaining $<5\%$ was unknown (Unitex 2006), we analyzed Uniplex FRP-45 by GC-TOF MS and Q Exactive high-resolution mass spectrometry. We conclusively identified EH-TBB ($<5\%$) in Uniplex FRP-45 (Supplementary data, Figures S1–S2). By contrast, we did not detect DEHP by TOF mass spectrometry. However, we detected [EH-TBB]⁺ and [DEHP]⁺ mass transitions in the standard solutions of EH-TBB, BEH-TEBP and Uniplex FRP-45 on a Q Exactive high-resolution mass spectrometer (Supplementary data, Figures S2 and S3). Because DEHP is a known ubiquitous contaminant in the laboratory environment, we could not rule out that detection of DEHP in Uniplex FRP-45 resulted from the presence of DEHP in laboratory reagents and supplies, or in the analytical apparatus (Koch and Calafat 2009).

We unambiguously identified TBPA and TBBA in the urine and serum of adult female Sprague–Dawley rats administered a single gavage dose (500 mg/kg bw) of Uniplex FRP-45 from the fragmentation patterns and retention times of authentic standards (Figs. 1, 2). This dose, much higher than expected environmental exposure levels from normal use of products containing non-PBDE flame retardants, was selected to ensure the formation of metabolites in sufficient amounts for their mass spectral identification. We did not detect TBPA or TBBA in the urine collected 24 h before dosing or in the serum of the three rats not dosed with Uniplex FRP-45 (i.e., “controls”).

The urinary and serum levels of TBPA and TBBA were highly correlated ($r > 0.9$, $p < 0.001$, Fig. 3). Interestingly, the mean levels of TBBA, a known in vitro and in vivo

metabolite of EH-TBB (Roberts et al. 2012), in urine (45.6 mg/L) and serum (1.2 mg/L) were higher than TBPA (0.5 mg/L in urine, 0.05 mg/L in serum), the expected hydrolytic product of BEH-TEBP (Table 2). The larger size and lower solubility of BEH-TEBP compared to EH-TBB (estimated log K_{ow} = 8.8 for EH-TBB vs. 12.0 for BEH-TEBP (US EPA 2014) may have contributed to TBBA and not to TBPA being the major metabolite detected in urine and serum. We hypothesize that because of its relatively low solubility and high molecular weight, BEH-TEBP may excrete preferentially unchanged in feces. Regrettably, we did not collect feces for this experiment.

Preliminary data suggested the presence of conjugates of TBBA and TBPA in urine (data not shown). However, because of the study design, we could not rule out hydrolysis of the conjugates during the 24-h collection period, while the animals were kept at room temperature. Therefore, additional experiments would be needed to determine the extent of conjugation. Although all rats were given the same dose, the levels of TBBA and TBPA in urine and serum varied widely (Fig. 3; Table 2). The variability in levels of these metabolites between dosed rats has previously been observed in rats dosed with phthalates (Calafat et al. 2006; Silva et al. 2012). Such variability could be explained by the expected inter-individual differences in absorption and metabolism of Uniplex FRP-45, as well as by differences in water intake of the animals during the experiment. Of note, similar variability was present even after correcting the concentrations of TBBA and TBPA for the dilution of the urine using specific gravity (Table 2). Some of this variability might have also been related to the analytical method used for quantification because D₄-MEHHP, not the corresponding isotopically labeled analyte, was used as the internal standard.

We also measured urinary MEHHP and MECPP, two metabolites of DEHP, the non-brominated analog of BEH-TEBP. Both MEHHP and MECPP mean levels were higher in dosed compared to control rats: MEHHP (0.30 ± 0.15 mg/L vs. 0.004 ± 0.002 mg/L) and MECPP (0.41 ± 0.25 vs. 0.0051 ± 0.0005 mg/L). These findings suggest that all rats (dosed and controls) were exposed to the ubiquitous environmental contaminant DEHP. Further, both MEHHP and MECPP levels correlated well with TBBA in the dosed animals ($R^2 > 0.88$; Supplementary data, Figure S4), but we did not identify tri-, di-, or mono-brominated intermediate debrominated metabolic products in dosed rats. Taken all of these findings together, we hypothesize that in the dosed animals, DEHP metabolites formed from DEHP present as a minor contaminant in the administered Uniplex FRP-45 rather than from debromination of BEH-TEBP in the rats, even though we could not conclusively establish whether the commercial mixture contained DEHP. Interestingly, although BEH-TEBP is structurally similar to DEHP, we did not detect BEH-TEBP oxidative metabolites similar to those formed by DEHP in urine of dosed rats.

Conclusions

In summary, we identified for the first time TBPA as an in vivo urinary and serum metabolite of BEH-TEBP in Sprague–Dawley rats. Unexpectedly, we also detected TBBA, a known metabolite of EH-TBB, at concentrations higher than TBPA even though BEH-TEBP was the main component of the flame retardant mixture used and EH-TBB was only a minor constituent. Our findings suggest that TBBA may serve as a biomarker of exposure to

select non-PBDE flame retardant mixtures containing BEH-TEBP and EH-TBB. TBPA alone may also serve as a specific biomarker of exposure to BEH-TEBP, but especially at occupational exposure levels. Additional research would provide valuable information regarding the composition of Uniplex FRP-45 and other flame retardant mixtures to better understand the in vivo toxic kinetics of their active components. These data, in turn, will be important to better understand the extent of human exposure to these mixtures and their potential impact on human health.

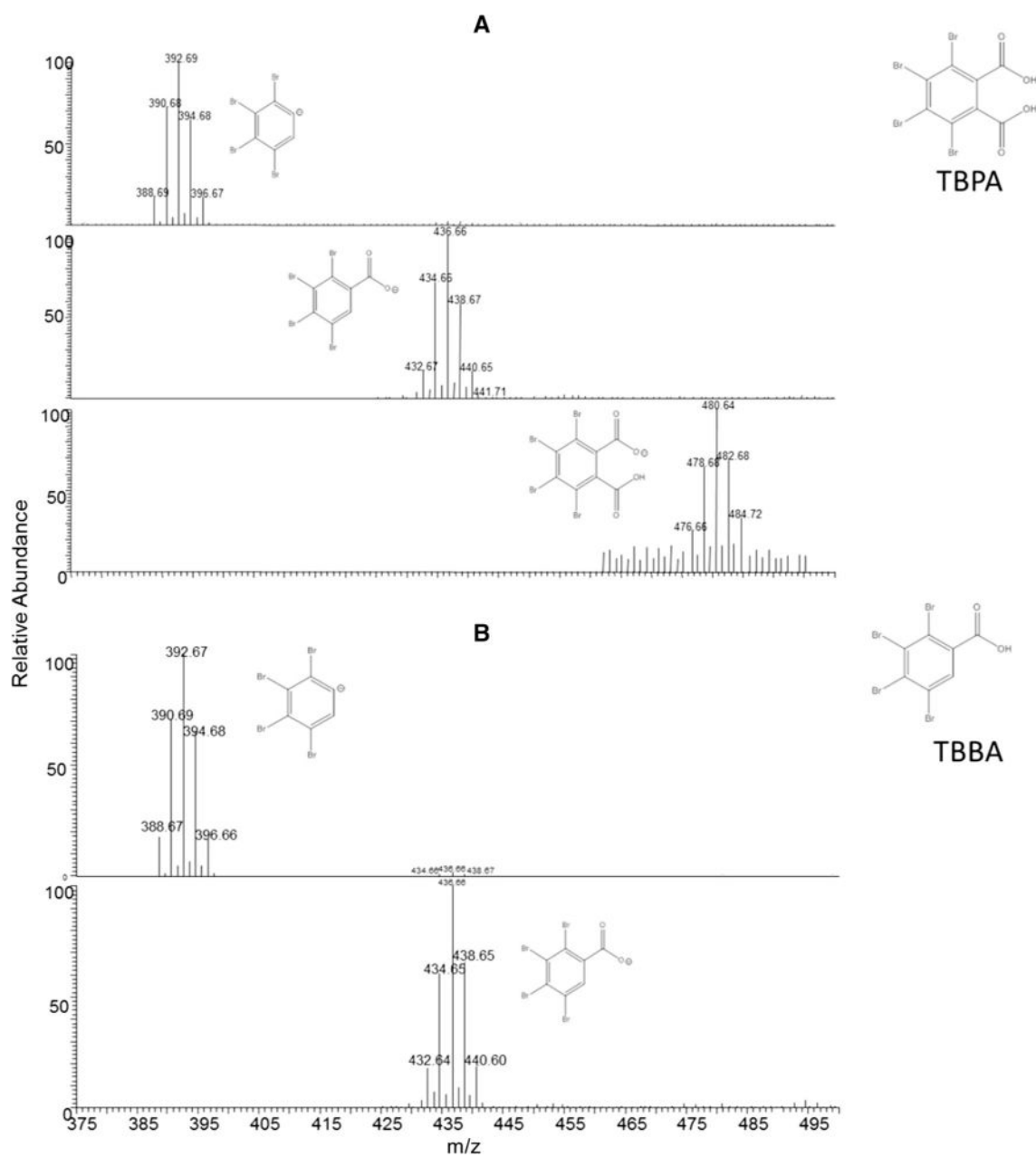
Supplementary Material

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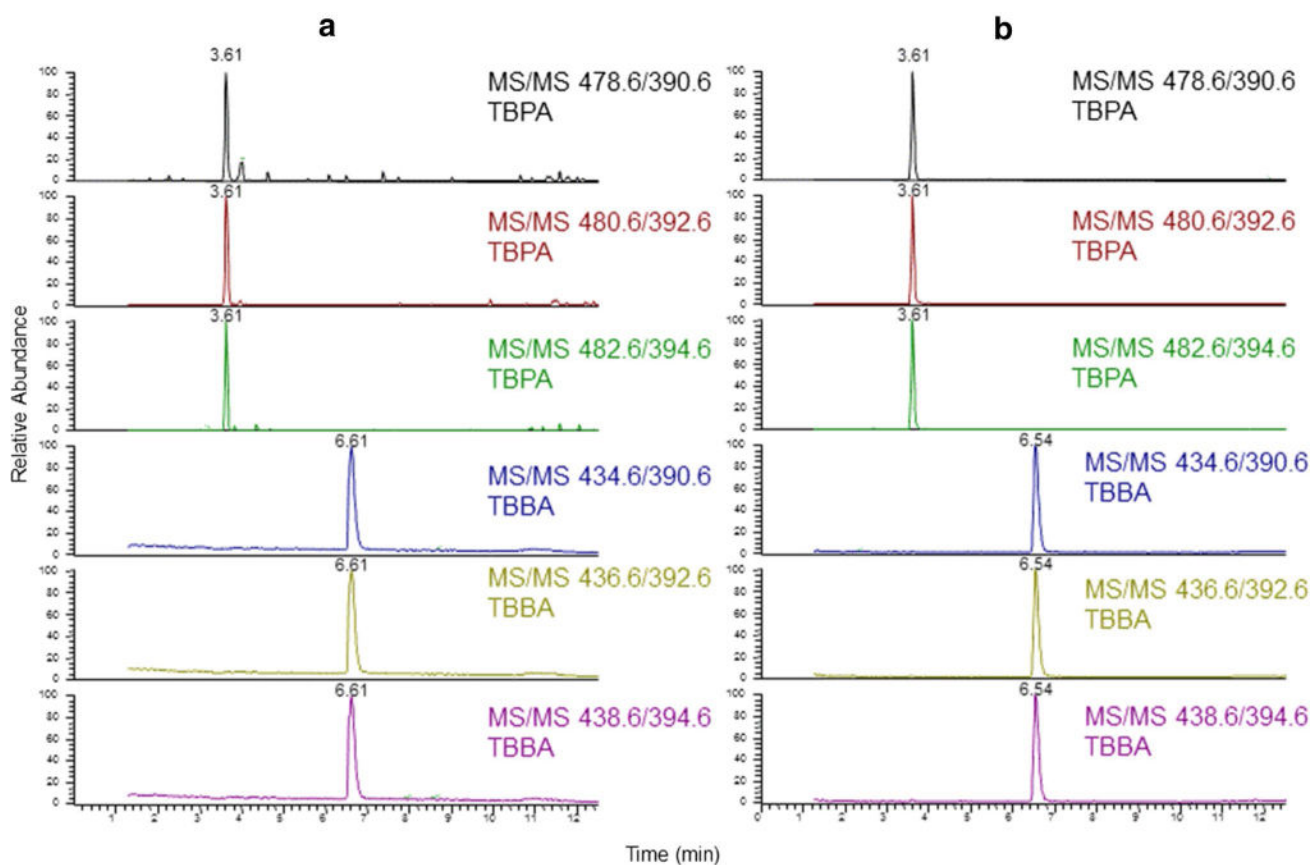


Fig. 2.

Chromatographic separation of tetrabromo benzoic acid (TBBA) and tetrabromo phthalic acid (TBPA) in **a** dosed rat urine, **b** standard solution containing TBBA and TBPA. Similar separation was observed in serum. TBBA and TBPA were not detected in the serum or urine of control rats

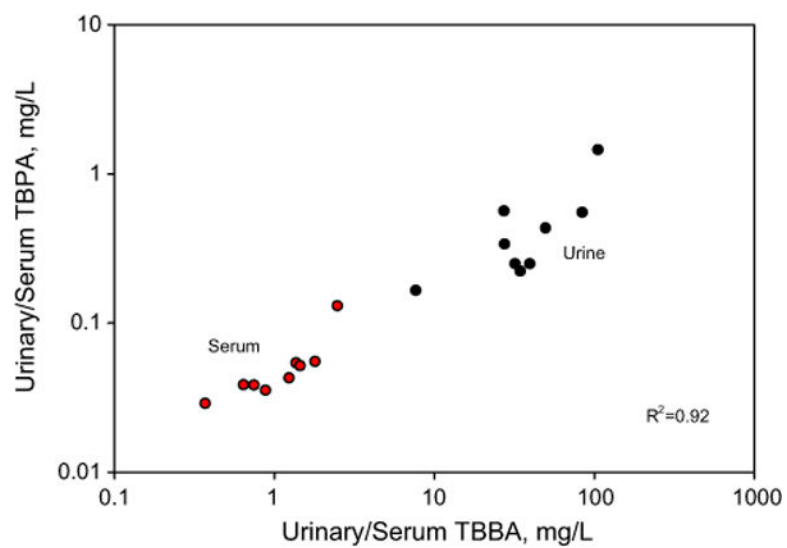


Fig. 3. Correlation plot of tetrabromo phthalic acid (TBPA) and tetrabromo benzoic acid (TBBA) in urine and serum of orally dosed female Sprague–Dawley rats. Each dot represents one rat

Chromatographic and mass spectrometric specifications used for measuring tetrabromo benzoic acid (TBBA) and tetrabromo phthalic acid (TBPA)

Table 1

Analyte	Quantitation transition (m/z)		Confirmation transition (m/z)		Retention time (min)
	Precursor	Product	Precursor	Product	
TBBA	436.6	392.6	438.6	394.6	6.5
TBPA	480.6	392.6	482.6	394.6	3.6
			436.6	392.6	

Collision energy 12 eV. D4-MEHHP, used as the internal standard, eluted at 4.3 min

Table 2

Urinary and serum levels of tetrabromo benzoic acid (TBBA) and tetrabromo phthalic acid (TBPA) in rats ($N = 9$) after oral gavage administration of Uniplex FRP-45 (>95 % BEH-TEBP, 500 mg/kg bw)

	<u>Mean urinary Levels \pm SD^a (mg/L)</u>		Mean Serum Levels \pm SD ^a (mg/L)
	Unadjusted	SG ^b adjusted	
TBPA	0.5 \pm 0.4	0.45 \pm 0.37	0.05 \pm 0.03
TBBA	45.6 \pm 30.8	43.8 \pm 29.2	1.2 \pm 0.7

^aSD standard deviation. The urinary and serum levels of TBPA and TBBA were <LOD in all controls ($N = 3$). LODs were 0.4 ng/mL for both compounds. TBBA and TBPA were not detected in urine collected 24 h before dosing

^bSG specific gravity